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Phenological and genetic characterization of Mediterranean plants at the peripheral range: the case of *Cistus albidus* near Lake Garda

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ABSTRACT

The potential effects of climate change on the distribution of Mediterranean plants at their peripheral range make them extremely vulnerable to environmental fluctuations. The northern-most Italian population of *Cistus albidus* provides an outstanding case for study, as it grows at the extreme northern edge of its range and because phenological data have been available since the early XIX century.

In the period 2012–2017, we analysed the population demography and spatial structure, growth and flowering phenology, and genetic variability to understand the history, current state and possible evolution of this population. Lastly, the population data were tested for association with environmental factors.

The population studied consists of plants on average 10 years old, showing the highest growth rates in spring and autumn. Anthesis starts in April and ends in early June and is significantly dependent on temperature. High levels of genetic variability are present (average $He = 0.471$), with heterozygote excess detected at four loci out of five. Bayesian analysis does not support any genetic structuring of the population.

The aggregated pattern in the distribution of individuals and their phenological traits, together with the absence of genetic structuring, seem to suggest that this extreme geographical population is not currently endangered.

1. Introduction

Peripheral populations *sensu lato* are those occurring in geographically and/or ecologically marginal areas, with respect to more central populations (Lesica and Allendorf, 1995). They can be distinguished into geographically marginal (= peripheral) populations, which are generally separated from central ones by a space of variable extent where the species under consideration is currently absent (for example, because of climatic variations that provoked range fragmentation), and ecologically marginal populations, which colonise areas at least partially different from the central ones from a biotic and abiotic viewpoint (Soulé, 1973; Shumaker and Babbel, 1980). However, geographically marginal populations are not rarely also ecologically marginal: they are often ubicated in sites that only partially recall the environmental conditions and habitats typical of the species (e.g. Madeira de Medeiros et al., 2018). Owing to their small size and scarcity of suitable environments, peripheral and isolated populations of plant species often show peculiar characteristics, such as fragmentation into subpopulations and/or alterations in the reproductive cycle, an increase in agamic propagation, autogamy and self-compatibility (Kjørup-Rasmussen and Kollmann, 2008). As a consequence, they often have a lower level of genetic diversity compared to central populations (Lammi et al., 1999; Beatty and Provan, 2011; Assis et al., 2013). This is the case for many species of the Mediterranean basin, one of the global biodiversity hotspots (Myers et al., 2000), which are scattered in small and isolated subpopulations or only limited to a few places (Raimondo et al., 2012; Coppi et al., 2014; Jiménez et al., 2017). The peculiar life-cycle and reduced genetic variation of peripheral populations make them vulnerable to even small environmental variations. Global climate change will have a particularly strong effect in the Mediterranean area

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(Gilpin and Soulé, 1986; Giorgi and Lionello, 2008; Bellard et al., 2012), thus making the study of peripheral populations of great interest for conservation purposes.

As a response to the selective pressures to which their populations may be subjected, plants show a certain phenological plasticity and the capacity to change life history traits in order to increase survival chances in extreme habitats or in conditions of range marginality, i.e. at the limit of the environmental conditions that a species is able to tolerate (Safriel et al., 1994; Abeli et al., 2014). Life cycles, different phenotypes resulting from the interaction between the genotype and the environment, and combinations of different reproductive modes by a given species in conditions of peripheral rarity, are some of the adaptations observed in various species typical of arid climates, e.g. *Anastatica hierochuntica* L. (Hegazy and Kabiel, 2010), *Senna candolleana* (Vogel) Irw. & Barn. (Lázaro-Nogal et al., 2015) and *Sedum hispanicum* L. (Pezzi et al., 2017). One of the most significant effects observed in marginal, isolated and fragmented populations is the alteration of parameters related to reproductive biology (Jones et al., 2001; Jump and Woodward, 2003; Hampe, 2005), such as fruit and seed production and seed viability (e.g., Sulis et al., 2017; D'Antraccoli et al., 2018). Peripheral populations can also show more genetic variation and be more resilient than central ones to environmental extremes and changes (Safriel et al., 1994). That said, peripheral populations are of great importance from a conservation standpoint, because of their evolutionary potential (Abeli et al., 2014), ultimately based on the degree of their genetic divergence from other conspecific populations, namely core-range ones (Lesica and Allendorf, 1995), their ecological features and population dynamics (Hampe and Petit, 2005). A very interesting case study in this regard is *Cistus albidus* L. in north-eastern Italy, where this shrub lives at the very margin of its range and for which phenological data have been available since the beginning of the XIX century.

C. albidus is a small evergreen shrub, on average 30–100 cm high, with coriaceous and tomentous leaves and hermaphroditic, actinomorphic, pentamerous flowers. On the plants, the flowers last on average between 12 and 24 hours (Bosch, 1992; Blasco and Mateu, 1995). The floral bud development takes place from March onwards; anthesis generally occurs between early April and late May (Bosch, 1992; Cabezudo et al., 1992). Its fruits are capsules containing numerous small (1–1.5 mm) seeds (Rizzotto, 1979; Demoly and Montserrat, 2005; Pignatti et al., 2017). Native to the western Mediterranean basin, it is found in Portugal, southern Spain (where it is quite common), the Balearic Islands, Morocco, Algeria (Demoly and Montserrat, 2005), southern France and (sporadic) Corsica (Tison and de Foucault, 2014), northern and central Italy and Sardinia (Pignatti et al., 2017). It is a steno-Mediterranean species, which grows spontaneously in garrigues, maquis and arid places between 0 and 1400 m a.s.l., in areas close to the sea or inland, where winter is mild and summer is warm. It requires rocky and calcareous substrates (Rizzotto, 1979; Demoly and Montserrat, 2005). Like other congeneric species, it is a vigorous colonizer of degraded and disturbed zones, where wildfires are frequent (Corral et al., 1990; Herrera, 1992).

Vegetative reproduction is based on two types of lateral shoots: dolichoblasts, produced from April to August and from December to February, and brachyblasts, produced during the whole year (Cabezudo et al., 1992). Flowering phenology has been studied under controlled conditions and in its natural South-Mediterranean habitat (Bosch, 1992; Cabezudo et al., 1992; Herrera, 1992; Blasco and Mateu, 1995), whereas the case of range marginality has never been analysed. Reproduction begins when the plants are 1–2 years old and peaks at the age of 5. On average, plants live for about 14 years (Roy and Sonié, 1992).

Pollination is primarily mediated by Apoidea (Blasco and Mateu, 1995), and the species is partly self-incompatible (Blasco and Mateu, 1995). Fructification takes place from May to August. Seed production is abundant (30–70 seeds/capsule), generally from August to December (Cabezudo et al., 1992; Blasco and Mateu, 1995). After shedding, the seeds remain near the parent plants; germination is epigeous (Rizzotto, 1979) and requires heat. Like other congeners, *C. albidus* is an active pyrophyte (Knapp, 1962; Montgomery and Strid, 1976). In fact, heat causes the rupture of the hard seed tegument and allows water absorption (Corral et al., 1989), thus indirectly favouring germination. Regeneration is rapid in the first 5 years after a wildfire and then greatly diminishes (Roy and Sonié, 1992).

In Italy the species is very rare and generally forms small, scattered populations. In this work we investigate the ecological and genetic aspects of an isolated population of *C. albidus* in the eastern side of the Lago di Garda. This population is of great phytogeographical importance: it is the northernmost of the entire *C. albidus* distribution known today (Pignatti et al., 2017), and may probably be, in turn, a relict population of ancient warmer periods.

By analysing demography, phenology, seed features and germination and by assessing basic population genetic parameters, our aims are both to improve the bio-ecological knowledge of this species at its peripheral range, and to understand whether current environmental trends are carrying a risk of extinction for the studied population.

2. Materials and methods

2.1. Study area

The population of *C. albidus* analysed in this work (Fig. 1) is found in Italy, on the eastern side of the Lago di Garda on a site named Le Prandine (Lat. 45° 37′ N, Long. 10° 42′ E), which falls into an area included in the Natura 2000 network as a Site of Community Importance (IT3210004 "Monte Luppia e P.ta San Vigilio"). The first mention of *C. albidus* in this area was made by Ciro Pollini in 1822, who greatly stressed the high abundance of the species in the collection place (Pollini, 1822). Many other mentions followed in subsequent decades, made by numerous botanists and plant collectors who visited the same population (e.g. Parlatore, 1872; Goiran, 1900; Béguinot, 1905).

The population of Le Prandine is made up of an aggregate of eight nuclei ranging from 200 to 250 m a.s.l., mainly exposed to WNW. *C. albidus* grows in clearings and in open areas of maquis, in which woody vegetation cannot establish permanently due to the stony and shallow soil. It co-occurs with herbs in quite sunny contexts, in full sun at times, but disappears in shady areas and where the maquis tends to close in. The surrounding vegetation consists of woodlands of *Quercus pubescens* Willd., *Fraxinus ornus* L. and *Ostrya carpinifolia* Scop., together with some Mediterranean species such as *Quercus ilex* L., *Pistacia terebinthus* L. and *Cotinus coggygria* L.; these formations are mixed with sparse semi-arid grasslands (*Festuco-Brometea*) with xerophilous and petrophilous species such as *Eryngium amethystinum* L., *Globularia bisnagarica* L., *Plantago holosteum* Scop., *Petrorhagia saxifraga* (L.) Link., and *Stipa pennata* L. Most of the thermophilous species growing here (particularly *Q. ilex*) are believed to be relicts of the vegetation of warmer post-glacial (anathermal) epochs (Bianchini, 1974; Brullo and Guarino, 1998; Biondi et al., 2003).

The main focus of the paper will be on the plants of nucleus 1, one of the largest nuclei and the one with the highest number of plants.

2.2. Population spatial structure and demography

During 2012, we counted plants older than three years, which is approximately the onset of fertile age, and all plants were georeferenced. The survey was repeated in 2017, also recording dead plants. Then, we calculated annual mortality and regeneration percentage rates. We measured trunk diameter, at a height of 3 cm above ground level, in living plants and in 24 dead not decayed plants collected during the 2017 survey. The number of annual growth rings was counted in a cross-section of the stem base of these dead plants. The

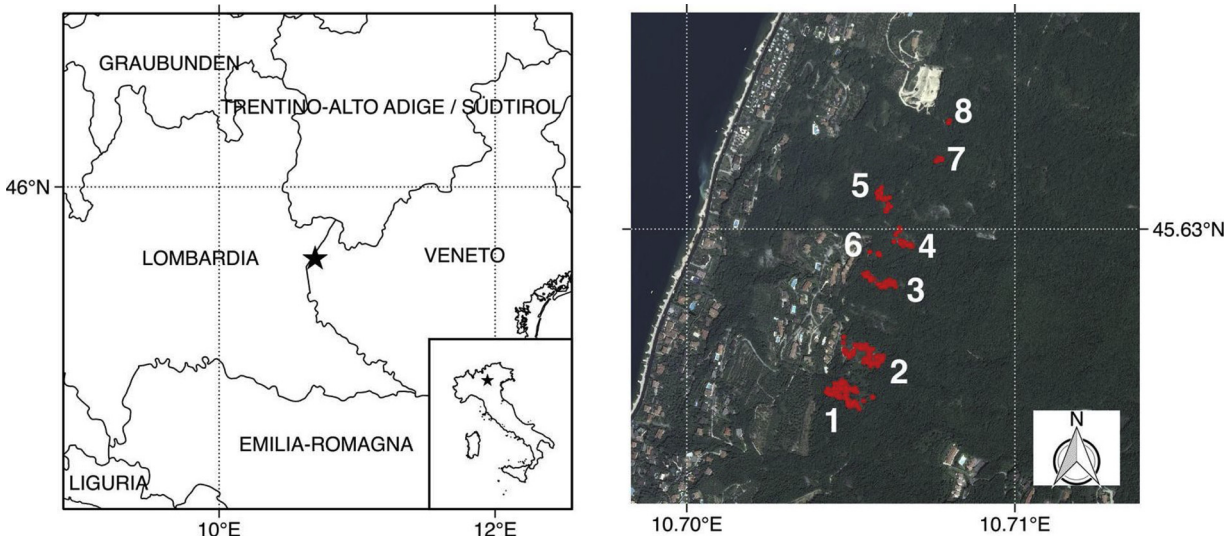

Fig. 1. Location of the study area (Lat. 45° 37′ N, Long. 10° 42′ E) and spatial distribution of the population nuclei. For further details see also Table 1.

diameter was then used as a predictor (independent variable) of the age (dependent variable), using a regression function in order to estimate the age of the living plants in nucleus 1.

Based upon the georeferenced data for individuals, the area of the whole population and of each nucleus was calculated by the convex hull method. The aggregation of the individual pattern was calculated with the Clark and Evans (1954) aggregation index *R* corrected for edge effects with the cumulative distribution function method (Baddeley and Turner, 2005), separately for all individuals and the individuals from nucleus 1 only. The calculations were performed with the R statistical software (R Core Team, 2015), using the functions *convexhull.xy* and *clarkevans.test* of the package *spatstat* v. 1.42–2 (Baddeley and Turner, 2005).

2.3. Seed features and germination

To highlight phenomena of reduced weight of seeds or low fertility, common in isolated and marginal populations (see e.g. Jacquemyn et al., 2001; Brys et al., 2004; Winter et al., 2008), germination tests were carried out on seeds collected from one capsule each of 56 plants randomly chosen from among adult plants of nucleus 1. Seeds were collected during August 2015 and stored dry for five months at 4 °C to simulate winter conditions and possibly trigger dormancy break. Another way of breaking dormancy is through mechanical manipulation of the seeds (Vuillemin and Bulard, 1981; Corral et al., 1990); therefore, in a second experiment, the seeds were slightly abraded by sand before germinating. In fact, in Cistaceae the seed dormancy is typically physical (Baskin and Baskin, 2004; Pérez-García et al., 2008): there is no specific dormancy mechanism exceeding the impermeability of the tegument and the slow germination rate of the seeds that underwent the tegument rupture (Thanos et al., 1992). The seeds were placed in phytotron for six weeks (photoperiod of 12 h light/dark, 15 °C), in pressed peat pots with a growth terrain made of potting soil (80%) and perlite (20%). The germination percentage was calculated following the procedure adopted by Roy and Sonié (1992) with minor modifications. We tested a total of 100 seeds and we classified as germinated all seeds that developed both a normal and abnormal seedling. We performed a weekly seed count for six weeks. To test variability in seed weight between different individuals we weighed 100 seeds for each of the 56 mother plants.

2.4. Growth phenology

In order to investigate the vegetative fitness of the individuals and the effect of the proximity to other plants on growth and drought sensitivity, during March we measured the plant height and crown length and width of each individual of nucleus 1 (crown width was expressed as the average between the longest crown axis and its orthogonal through the central mass of the crown itself). We then categorized the crown shape, whenever possible, based on the ratio crown length/width (Cl/Cw; levels: 1 = vertically oblong [Cl/Cw ≥ 1.25], 2 = rounded [Cl/Cw < 1.25]) and the degree of foliage desiccation (0 = no desiccation, 1 = desiccation < 20%, 2 = desiccation between 20 and 50%, 3 = desiccation > 50%; see also Table S1 in Electronic Supplementary Material).

A χ^2 tests were carried out to study the relationship between different phenological traits and percentage of forest canopy cover (levels: 0 = no coverage, 1 = coverage < 20%, 2 = coverage between 20 and 50%, 3 = coverage > 50%).

Then, spatial autocorrelation in the plant size – that is the correlation between trunk diameter, plant height and crown size due to the plant location – was calculated with the Moran's I (Moran, 1950) index, by using the function *Moran.I* of the package *ape* v. 5.1 (Paradis et al., 2004) of the R statistical software (R Core Team, 2015).

Finally, to understand growth dynamics in the years 2016 and 2017, we measured shoot elongation on a random sample of 31 three to four-year-old plants within nucleus 1. For each sampled plant, we measured the apical shoot thrice a year, on March 21st, June 21st and September 21st, in order to evaluate growth variation in different seasons.

2.5. Flowering phenology

2.5.1. Current monitoring

Flowering phenology was monitored over four years (2013, 2014, 2016, 2017) for the entire anthesis period (from early April to early June), taking into account all plants of nucleus 1. Once a week the number of open flowers per plant was recorded. Based on these data, four key phenological parameters were obtained: flowering beginning (i.e. the first day with open flowers in the population), flowering peak (i.e. the day with the highest number of open flowers in the population), end of flowering (i.e. the first day when all flowers are withered) and flowering duration (i.e. the number of days between the beginning and the end of flowering).

2.5.2. Herbarium specimens

Owing to the lack of historical phenological series, we used herbarium specimens to expand the time span of phenological records. *Exsiccata* collected in the vicinities of the Lago di Garda, in years

preceding our monitoring, were searched for in all the 33 Italian university herbaria (see Taffetani, 2012 for a review) and the herbaria of the 5 principal natural history museums of northern Italy, i.e. the closest ones to the study area (MSNM, BOZ, MFU, HbBS, VER). The herbarium sheets were scanned at high resolution and sent to us (see Table S2 in Electronic Supplementary Material).

Phenological analysis was performed on specimens with a complete date of collection (day-month-year). The anthesis phenophases were then attributed depending on the presence of floral buds, open flowers, withering or fructification. Each specimen was attributed to the phenophase "beginning of flowering" if it showed only buds or buds with some open flowers; conversely, it was attributed to the phase "flowering peak", if it showed only open flowers or some buds together with open and withered flowers, according to Marcello (1935). Then, we used the date of collection of the specimen (written on the herbarium sheet label) as the day of the year (DOY) of occurrence of that flowering stage.

2.5.3. Data handling

For current data, the relationship between the day of occurrence (DOY) of the flowering stages (beginning and peak) and average temperatures and/or precipitation of the previous months (January to April) were statistically tested, as already done for example by Menzel et al. (2006). We used a multiple linear regression analysis, processing the phenological variable "date of flowering" as a dependent variable, whereas the flowering stages ("phen", expressed as beginning = 0 or peak = 1), average temperatures (T) and rainfall (RAIN) were used as predictors (independent variables). Significant variables were chosen through backward elimination for both current and herbarium data. Data of precipitation and temperature were obtained from the geographical coordinates of the sampling site (see Brunetti et al., 2012). This analysis was performed considering four different periods: January-April (JA), February-April (FA), March-April (MA) and April (A).

2.6. Genetic analysis

A total of 214 plants were sampled from nucleus 1. The collected leaves were frozen at −80 °C and total genomic DNA was extracted by grinding the frozen leaves in a mortar and liquid N_2 using the DNeasy Plant Mini Kit (Qiagen, Italy), according to the manufacturer's instructions.

Four pairs of heterologous microsatellite (SSR) primers developed by Astuti et al. (2017) for *Cistus laurifolius* L. and successfully tested for their ability to amplify *C. albidus* were used (*Cislau1*, *Cislau11*, *Cislau12* and *Cislau14*). Primer sequences and annealing temperatures are reported in Electronic Supplementary Material 3.

SSR reactions were carried out in a total volume of 20 µl, containing 1× PerfectTaq (5PRIME®) buffer, 0.5 mM $MgCl_2$, 2 µM of each dNTPs, 0.25 µM of each forward and reverse primer, 20 ng genomic DNA and one unit of Taq polymerase (5U/µl) (PerfectTaq, 5PRIME®). PCR amplifications were performed using an EpGradient thermal cycler (Eppendorf®) under the following conditions: an initial cycle at 95 °C for 3 min; followed by 30 cycles at 95 °C for 1 min, annealing temperature (Ta) for 1 min, 72 °C for 2 min; and a final extension at 72 °C for 7 min. The PCR products were analyzed by capillary technology at Macrogen®. The raw data obtained were analysed and checked using Peak Scanner 2.0 software (Applied Biosystems) to score the single-plant genotypes. Amplification patterns for the *Cislau14* primer were consistent with the presence of a second locus: this prompted the following analyses to be carried out as two separate loci, *Cislau14a* and *Cislau14b*.

Allele frequencies and observed and expected heterozygosities were estimated at each locus. Fisher's exact test using the Markov Chain algorithm (Guo and Thompson, 1992) was used to test for deviations from Hardy-Weinberg equilibrium. Weir and Cockerham's, (1984) estimator of F_{IS} was used to assess the degree of inbreeding. For these analyses we used GenePop 4.0 (Rousset, 2008) and Genetix 4.05.2 (Belkhir et al., 1996).

To investigate the presence of sub-structuring in the population, an estimate of *K*, the most probable number of ancestral genetic pools present, was done by using the Bayesian clustering method implemented in STRUCTURE 2.3 (Pritchard et al., 2000). Ten independent runs were performed for each *K* between one and six, with a no admixture model, 50,000 MCMC iterations and a 10000-iterations burn-in period. We then tested for the most plausible number of clusters *K* using the approach based on Δ*K* developed by Evanno et al. (2005), by using Harvester (Dent and vonHoldt, 2012). Results from each batch of ten runs from the same K were then summarised using CLUMPP (Jakobsson and Rosenberg, 2007) and the results were graphically processed using DISTRUCT (Rosenberg, 2004), in particular the online resource CLUMPAK (Kopelman et al., 2015).

3. Results

3.1. Population spatial structure and demography

The total surface of the population of *C. albidus* was 72,000 m^2 and its total number of individuals ranged from 498 plants in 2012 to 455 in 2017 (Table 1). The eight nuclei differed in size, from 51 m^2 for nuclei 6 and 7 to 3863 m^2 for nucleus 1. The number of plants per nucleus was also highly variable, from a minimum of 3 plants for nucleus 8 in 2012 to 249 plants for nucleus 1 in 2017. The distance between each neighbouring nucleus varied from 47 to 143 m. Based on the results of the Clark-Evans test, the individual pattern was aggregated both at the population level ($R = 0.26$, $p = 0.001$) and in nucleus 1 ($R = 0.50$, $p = 0.001$). Measured over five years, the annual percentage of deaths (mortality rate) on all nuclei varied from 2.3 to 7.6%, whereas the percentage of seedlings (regeneration rate) ranged from 6.1 to 18.8%. The value of the two rates, calculated on the total number of individuals, was 6.1 and 9.0%, respectively (see Table 1 for further details).

Table 1
Extent and demographic profile of nuclei of *C. albidus* in the Le Prandine population. Annual regeneration and mortality rates are also provided. Data concerning nucleus 2 are available only for the year 2012, since it was heavily impacted by human activities in the following years. Therefore, the regeneration and mortality rates of the whole population do not include nucleus 2 (plants in 2012 are counted as 393). The total area results from a convex hull which envelops all the nuclei in 2012.

Nucleus	Area (m^2)	Plants (n) 2012	Plants (n) 2017	Death (n) 2017	Regeneration (n) 2017	Regeneration rate (%)	Mortality rate (%)
1	3863	234	249	92	107	8.9	7.6
2	3672	105	n.a.	n.a.	n.a.	n.a.	n.a.
3	1258	78	91	15	28	6.6	3.5
4	919	48	70	11	33	11.2	3.7
5	1022	20	19	7	6	6.1	7.2
6	51	5	12	1	8	18.8	2.3
7	51	5	9	2	6	17.1	5.7
8	< 1	3	5	1	3	15.0	5.0
All	72,000	498	455	129	191	9.0	6.1

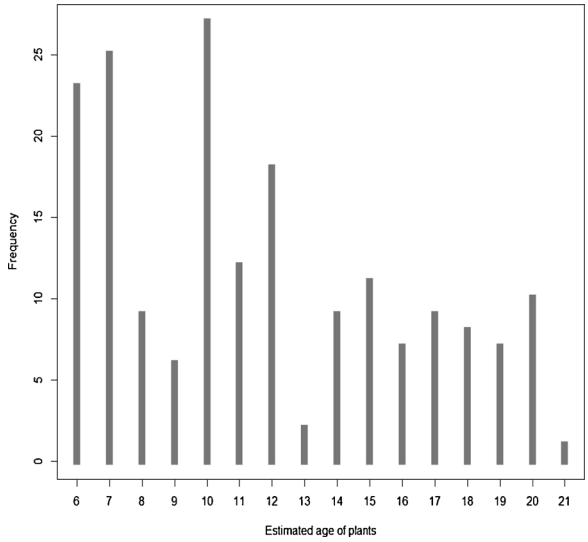

Fig. 2. Frequency of estimated age of living plants in nucleus 1. Age was estimated by measuring trunk diameter, as described in Section 2.1.

The best fitting linear regression between diameter of trunks and their age was a second grade polynomial (b_1 = 0.637, b_2 = -0.005). Using the above mentioned function, we estimated the age for 184 plants with diameter in the range 9.5–58 mm. Most of the plants were estimated to be around 10 years old, ranging between 6 and 21 years; however, a trunk of 81 mm of diameter and 24 years was found. It is therefore plausible that the oldest plants can reach ages of 24 years or slightly higher. The frequency distribution for estimated ages is shown in Fig. 2, which also evidences the relative paucity of plants between 8–9 and 13 years old.

3.2. Seed features and germination

The average number of seeds per capsule was 70. The weight of 100 seeds, averaged on 56 mother plants, was 102 mg (C.I. 95% = [98–106] mg). The percentage of seed germination was estimated at 8% (C.I. 95% = [2.7–13.3] %) for untreated seeds, but increased to about 75% for abraded seeds.

3.3. Growth phenology

All single plant data (here including the geographic coordinates) are reported in Table S1 (Electronic Supplementary Material).

Phenological traits measured in the plants of nucleus 1 were averaged and yielded the following results (95% confidence intervals in parenthesis): trunk diameter 20.7 mm (19.0–22.4), height 77.5 cm (74.6–80.4), crown diameter 67.9 cm (63.4–72.4). The distribution pattern of the values of diameter, height and crown size was clustered, with Moran's I ranging from 0.06 to 0.07 and higher than what is expected under spatial randomness.

On a total of 234 plants, 49% showed no desiccation, 28% low desiccation, 14% medium and 9% high; concerning crown shape, 180 plants (77%) were classifiable: 54% showed a vertically oblong shape and 46% rounded (see Material and Methods for details). The extent of canopy cover showed a significant negative relationship with the desiccation level of plants (χ^2 = 32.25, ν = 9, p = 1.8e-4), especially regarding the high-desiccation class, which showed a significant positive relationship with the loss of coverage by the canopy.

Measurements of shoot elongation are shown in Table 2. The periods of fastest growth were spring 2016 (69% of total elongation) and autumn 2017 (40% of total elongation).

Table 2
Seasonal growth of *C. albidus* in the Le Prandine population (nucleus 1). Seasonal growth was estimated on a random sample of 31 plants within nucleus 1, by measuring apical shoot elongation thrice a year. For further details on growth estimate, see Section 2.4.

	2016 growth (mm)			2017 growth (mm)		
	spring	summer	autumn	spring	summer	autumn
average	173	62	16	101	70	113
C.I. (95%)	151-195	50-75	12-21	90-113	48-92	94-132

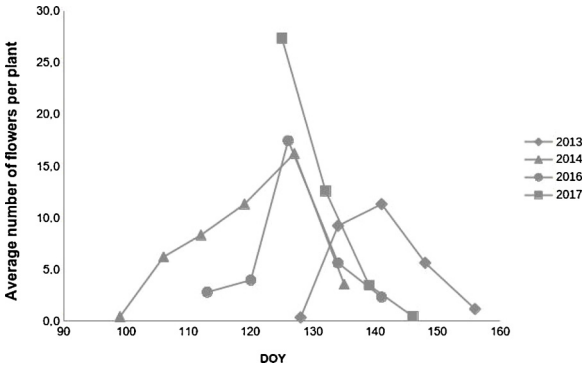

Fig. 3. Comparison of the four years of phenological observation on the anthesis of *C. albidus*. DOY: day of the year.

3.4. Flowering phenology

Between 2013 and 2017, 240 plants were sampled, for a total of 17,337 flowers. The flowering cycle of *C. albidus*, measured in the period 2013–2017, lasts 28–37 days and is comprised between April and early June. On average, the beginning of flowering is on DOY 113 (23 April), the peak of flowering is on DOY 130 (10 May) and the end of flowering is on DOY 145 (25 May). However, there are considerable variations from year to year (Fig. 3). During the peak of flowering, the average number of flowers per plant was 18 considering the entire period 2013–2017, variable from 11 in 2013 up to 27 flowers in 2017.

The regression models testing the relationship between DOY and temperature were significant, but not those with rainfall (Table 3). According to these results, the interval between the beginning and the peak of flowering is of about 16 days, whereas the flowering phenophases appear with an anticipation of about 8 days for every additional Celsius degree rise in the average temperature of the previous months.

The number of herbarium specimens seen was globally 41 (see Table S2 in Supplementary Material), but the phenological analysis was carried out exclusively on the 22 specimens with a complete date of

Table 3
Regression models for the relationship between the time of beginning of flowering (DOY) and environmental variables for *Cistus albidus* in the Le Prandine population (current monitoring data). Dependent variable: DOY = day of occurrence of the flowering stages (expressed in days of the year). Predictors: "phen" = flowering stages (expressed as beginning = 0 or peak = 1); T = average temperatures in °C of the months preceding flowering. The months considered were January (J), February (F), March (M), April (A), and the periods tested were JA (from January to April), FA (from February to April), MA (from March to April), A (only April). The regression models for rainfall are not shown, since they are never significant.

Time of the year	Regression model	p	R^2
January-April	DOY = 15.06 phen - 8.50 T_{JA} + 183.28	0.001	0.963
February-April	DOY = 16.84 phen - 7.44 T_{FA} + 183.80	0.002	0.958
March-April	DOY = 17.76 phen - 8.47 T_{MA} + 209.07	0.004	0.935
April	DOY = 14.76 phen - 18.59 T_{A} + 368.70	0.006	0.923

Table 4
Regression models for the relationship between the time of beginning of flowering (DOY) and environmental variables for *Cistus albidus* in the Le Prandine population (herbarium data). Dependent variable: DOY = day of occurrence of the flowering stages (expressed in days of the year). Predictors: "phen" = flowering stages (expressed as beginning = 0 or peak = 1); T = average temperatures in °C of the months preceding flowering. The months considered were January (J), February (F), March (M), April (A), and the periods tested were JA (from January to April), FA (from February to April), MA (from March to April), A (only April). The regression model of the last case was not reported in the table, since it was not significant. The regression models for rainfall are not shown, since they are never significant.

Time of the year	Regression model	*p*	R^2
January-April	DOY = - 18.35 T_{JA} + 279.86	0.026	0.529
February-April	DOY = - 24.16 T_{FA} + 351.50	0.008	0.661
March-April	DOY = - 19.37 T_{MA} + 344.59	0.013	0.607

collection. The presence of flowers in June (i.e. in the very last part of the flowering season) was recorded three times: *Leg.* Pollini, 6–1824, in BOLO; *Leg.* Manganotti, 20-6-1846, in VER – DOY 171 –; *Leg.* Di Carlo, 21-6-1984, in VER – DOY 173 –.

Regression analyses on the herbarium data confirmed the general findings obtained by current monitoring data: temperatures were significant in most cases (except April), whereas phenological stage and rainfall coefficients were never significant (Table 4). The anticipations of the phenological response are much higher (16–21 days) than those observed in the case of currently living populations.

3.5. Genetic analysis

Genetic variability in nucleus 1 of the Le Prandine population was high, as indicated by the data in Table 5. The average number of alleles per locus was 3, total observed heterozygosity H_o = 0.622 and total expected heterozygosity H_e = 0.471.

Four loci out of five were not at Hardy-Weinberg equilibrium, and in three cases departure from equilibrium was due to heterozygote excess.

In the Bayesian analysis aimed at finding *K*, no genetic sub-structuring was evident. In fact, the values of lnP(*D*) (Electronic Supplementary Material 4) show a monotonous decrease, evidence for non-structuring of the population, as confirmed also by the ΔK statistics and by the visual analysis of bar plots under different *K* (Electronic Supplementary Material 5).

4. Discussion

The research here presented provides a picture of the flowering phenology of *C. albidus* in conditions of range marginality and reproductive isolation, together with a preliminary assessment of the degree of genetic variability harboured by such a population.

4.1. Population spatial structure and demography

Although Müller et al. (2014) ascribed a maximum age of 25 years to the effect of fire events in their study site, our results support a life span around 21 years or older for *C. albidus* in Le Prandine, even in the absence of such disturbing phenomena. The highest age frequency is centred around 10 years, that is approximately half the estimated maximum age. The regular decrease in the frequency of older class ages leads us to suppose that the natural process of ageing, and not anthropic or evolutionary processes involving the overall ecology of the site, shapes the actual demographic status of nucleus 1. However, gaps in frequency of age for plants of 8 and 12–13 years old are present. This situation may be related to environmental phenomena acting episodically on seed recruitment. Similar results were obtained by Roy and Sonié (1992) for *C. albidus* and *C. monspeliensis* in southern France, but in such cases the lack of some age classes was due to previous fires or climate constraints. We could speculate about some autotoxicity phenomena (as demonstrated by Robles et al., 1999, specifically for *C. albidus*) or other endogenous factors, that might provoke periodical death waves in adult individuals (e.g. Hernández Plaza et al., 2012; Mazzoleni et al., 2015; Cesarano et al., 2017; Vincenot et al., 2017). Nonetheless, individuals are spatially aggregated as expected by facilitative processes in the first phases of seedling development and local dispersal of seeds (Tirado and Pugnaire, 2003; Lara-Romero et al., 2016).

The equilibrium state is dynamic: this fact is shown by the relatively high percentages of dead and young plants recorded in all nuclei (years 2012–2017) and by the strong ability to promptly substitute dead plants with young ones as a common trait of all nuclei. In some cases, we could hypothesize that this phenomenon may be enhanced by the reduction in canopy density in the surrounding vegetation, similarly to what we observed when *Ostrya carpinifolia* (which composes the woodlands surrounding *C. albidus*' nuclei) underwent a reduction in canopy density (data not shown). Our data underline how various nuclei have different demographic characteristics and that larger nuclei (nucleus 1 in particular) appear to be more stable than smaller ones.

Table 5
Genetic variability of the Le Prandine population. Observed (H_o) and expected (H_e) heterozygosities and F_{IS} values for the five loci and for the whole population are reported. *p* = probability that F_{IS} = 0.

Locus	H_o	H_e	F_{IS}	*p*
cislau1	0.800	0.489	−0.652	$< 10^{-4}$
cislau11	0.995	0.568	−0.750	$< 10^{-4}$
cislau12	0.460	0.638	0.283	$< 10^{-4}$
cislau14a	0.198	0.178	−0.107	0.230
cislau14b	0.658	0.486	−0.350	$< 10^{-4}$
Total	0.622	0.471	−0.318	$< 10^{-4}$

4.2. Seed features and germination

The percentage of germination obtained (about 8%) is intermediate between those obtained in the congeneric *C. creticus* L. (3.4%, Stephanou and Manetas, 1998) and *C. ladanifer* L. (18.7%, Delgado et al., 2008). Both species present analogous adaptations to the Mediterranean environment, such as the presence of hard seed teguments, which is considered an adaptation to dry environments because the tegument needs to be removed before germination. Therefore, this typical xerophytic adaptation to a Mediterranean environment is maintained even at the northernmost range of the species (Trabaud and Oustric, 1989). However, the methodological differences among the experiments may have not negligible effects: our germination test was performed in phytotron, simulating a photoperiod and temperature typical of early spring, whereas the percentages reported by Stephanou and Manetas (1998) and Delgado et al. (2008) were obtained under natural conditions.

The average weight of 100 seeds (102 mg) is also similar to other results obtained on the same species for populations from NE Spain (Müller et al., 2014).

Concerning the experiment of tegument abrasion, our results are in line with what was found for other congeneric species: when seeds underwent mechanical scarification, in *C. symphytifolius* Lam. germination increased from 3 to 97% (Pérez-García et al., 2008), in *C. albidus* up to 97% and in *C. creticus* L. ssp. *creticus* even up to 100% (Thanos et al., 1992). Even if a satisfying explanation has not been found so far (Thanos et al., 1992), such pre-treatment might perhaps imitate, in some respects, what naturally occurs in the case of fires, that is the rupture of the seed tegument due to the heat, as observed specifically in *C. albidus*, whose germination rate increased from 7 to 65% if the seeds underwent a dry-heat pre-treatment (Escudero et al., 1997).

4.3. Growth phenology

The growth of *Cistus albidus* in the Le Prandine population shows the typical growth pattern of Mediterranean species, with a marked reduction during summer and notable growth during autumn (see Table 2). This is in line with previous studies made on the same species (Castro-Díez and Montserrat-Martí, 1998) and is noteworthy because the Le Prandine population is the northernmost of today's distribution (Pignatti et al., 2017). The values observed for several growth-related traits show both a wide variability for this character between individuals and an elevated plasticity of this species that appears to be able to re-modulate growth pace to cope with different climatic variations in different years. This is also indicated by the significant negative relationship found between degree of desiccation and canopy cover, which could probably be explained by supposing some specific adaptations to the local environment. Such adaptations would disadvantage the species in the case of too much wind, too much UV radiation and too scarce sunlight shielding, in contrast to what can be observed, for example, in Mediterranean France or southern Spain, where *C. albidus* is well adapted to grow in full sun (Demoly and Montserrat, 2005; Tison and de Foucault, 2014). Nonetheless, as already observed in the individual distribution, the phenological traits of the individuals of the Le Prandine population are spatially clustered, as expected in spontaneous tree and shrub stands, as a result of facilitative processes.

4.4. Flowering phenology

In the study site, *C. albidus* seems to maintain the same flowering rhythm as in the other parts of its range, including the most southern sites of the Iberian peninsula (Herrera, 1992; Blasco and Mateu, 1995; Villacampa et al., 1997). Anthesis mostly occurred in April-May and lasted on average about 30 days. However, the presence of flowers in June is possible, as we found both in the years of monitoring and in the herbarium samples, and is probably due to the particular meteorological conditions of those years. Our findings coincide with those of Castro-Díez and Montserrat-Martí (1998), who reported an anthesis period in April-May, and those of Villacampa et al. (1997), according to whom a first flowering could take place in March and the very last one even in late July. However, concerning the principal factors influencing the flowering timing, our results are in contrast with the observations made by Peñuelas et al. (2004), Prieto et al. (2008) and Gordo and Sanz (2010), who state that rainfall also has a significant role in determining the flowering time of Mediterranean species, even though it is not as important as temperature. In fact, in our study, there is no significant correlation with rainfall, whereas a strong correlation is present with the average temperatures of the period preceding the anthesis (especially from January-February to April). A similar result was observed considering the data from herbarium specimens.

The greatest differences between monitoring and herbarium data concern the phenological response: the herbarium data show a phenological shift much higher than that calculated on the monitoring data (16–21 days versus 8). To our knowledge, the comparison of monitoring data and herbarium data spanning almost 200 years has never been made before for any plant species. Even though the direct comparison of the results is biased by the small number of herbarium specimens and the large time-span considered, such a clear convergence in the results of the two data sets is worth noting, also because it corroborates the results themselves. We take this as indication that the conjoined use of herbarium and current monitoring data could be a cornerstone for studying population changes over long periods of time. In this regard, it is tempting to assume that the observed shift of flowering time (more than one week) reflects some actual changes at the species-environment interaction level.

In any case, we can state that this population of *C. albidus* shows a quite high sensitivity and a very high response to temperature, when compared to other species. In fact, the response to environmental factors is variable both among different species and even within the same species, as shown by Ibáñez et al. (2010). In a huge study considering hundreds of historical phenological series for 542 species in central Europe (Menzel et al., 2006), the values of regression coefficients indicated at most 4–5 days of anticipation for every Celsius degree rise in the average temperature of the previous months. The nearly double anticipation value we recorded for *C. albidus* (8 days) is possibly justified by the Mediterranean origin of the species: various authors noted that, during spring, the phenological response to the temperatures is more intense in warmer than in colder regions (Wang et al., 2015). Another explanation resides in the different time scale considered: Menzel et al. (2006) examined by default only the temperatures of the two months preceding anthesis, whereas our analyses took into account the temperatures starting from January. This may have an effect on the differences regarding the estimates of the regression coefficients. In fact, as demonstrated by the occurrence of flowering until late June, one must consider that the temperatures have a long time span to affect the flowering process, starting from January onwards. Again, data from herbarium specimens are interesting in this sense: as shown by our results, *C. albidus* is sensitive to quite low temperatures and seems to have quite a low thermal threshold (however, to ascertain this point a dedicated study would be necessary). This fact is uncommon in steno-Mediterranean species: it has been recorded, for example, in some cultivated species such as *Vitis vinifera* L. and *Olea europaea* L., which have a thermal threshold of 10 and 7–15 °C, respectively (Oliveira, 1998; Orlandi et al., 2005).

A strong response to temperature in the flowering time of *C. albidus* could have consequences on the species fitness, in the case of an acceleration of climate change. In fact, it is suspected that a connection exists between the decline or extinction of local populations and the reduced ability to vary anthesis times in response to climate changes (Bradley et al., 1999; Willis et al., 2010). Similarly, the colonisation success of various allochthonous species would be explainable thanks to a rapid response to weather variations (Willis et al., 2010; Wolkovich et al., 2013). In addition, variations in phenology may have notable impacts for biological conservation, potentially creating ecological mismatches between interacting species: this fact has been widely examined from a theoretical standpoint (see, for example, Miller-Rushing et al., 2010). Even if at present there is still insufficient experimental evidence concerning the real effects of the rupture of synchrony among diverse species, we may say that these mismatches lead to a decrease of the phenological overlap between plant and pollinator, which can cause a notable decrease in seed production, with obvious negative consequences for both species' survival in the long-term (Alexander et al., 2016). This was already observed in perennial species, such as *Corydalis ambigua* Cham. & Schltdl. (Kudo and Ida, 2013) and *Lathyrus littoralis* (Nutt.) Endl. ex Walp. (Olliff-Yang and Mesler, 2018).

4.5. Genetic variability

SSR markers revealed a good reservoir of genetic variability in the Le Prandine population of *Cistus albidus* (average H_e = 0.471, average number of alleles/locus = 3). In the only other paper dealing with genetic variability in *Cistus albidus* (Grant et al., 2006), based upon seven isozyme loci for three enzymatic systems, assessed in seven populations from Southern Spain, the same estimates were: H_e ranging from 0.16 to 0.53 (average = 0.40), average number of alleles/locus = 2.39. The estimates are remarkably close, also in the high levels of genetic variation (H_e = 0.53) found for the population of this study, which is comparable to that of Le Prandine from the point of view of their isolation. The Spanish populations differ from that of Le Prandine when inbreeding is taken into account. Average F_{IS} is 0.12 in Grant et al. (2006), thus there is an excess of homozygous plants, due to the extremely high value of 0.53 exhibited by a single population.

Our data are also in agreement with the results from other studies on *Cistus* species: in *C. salvifolius* – the very species for which the SSRs

used in our study were developed – Farley and McNeilly (2000) found for allozymes $H_e = 0.47$, the same value as our estimate, with an average number of alleles/locus = 2.09. In the study on *C. salvifolius* only 13 populations were analysed, meaning that our single population of *C. albidus* displays the same levels of genetic variability as *C. salvifolius* at the species level. The same holds true when looking at inbreeding levels, almost identical again, estimated by F_{IS} = -0.318 for the single population of *C. albidus* and F_{IS} = -0.31 for the thirteen populations of *C. salvifolius*. It appears that mechanisms exist within the genus to avoid homozygosity, as also indicated by the fact that almost identical estimates were obtained by two different classes of genetic markers.

However, when compared with an Italian population of another *Cistus* species, i.e. *C. laurifolius*, analysed with a set of SSRs partially overlapping with ours (Astuti et al., 2017), the differences are striking. In fact, lower genetic variability is detected ($H_e = 0.32$), but the main difference resides in the F_{IS} values, since in three out of four sub-populations studied, *C. laurifolius* presents an excess of homozygotes. Different age classes sampled and/or present in the *C. laurifolius* and in our *C. albidus* populations could explain this different behaviour, but effects of population fragmentation present in *C. laurifolius* and not in *C. albidus* could also be responsible.

Another study on *C. ladanifer* L. estimated even lower variability levels using both AFLPs and cpSSRs on 33 populations of the Iberian peninsula ($H_e = 0.098$ and 0.032, respectively – Quintela-Sabarís et al., 2012), but, in this case, the differences can be ascribed to the different kind of markers used; in fact, AFLP are dominant markers with a possibly high homoplasy and different mutation rate than SSRs, while cpSSRs, maternally inherited, have a smaller effective population size than nuclear SSRs.

No evidence of genetic erosion is therefore apparent for the Le Prandine population, leading us to infer that entomophilous cross-pollination is effective in counteracting possible adverse genetic effects due to isolation, such as inbreeding. Again, it must be borne in mind, however, that we used neutral genetic markers, so we cannot draw any conclusion about fitness levels.

5. Conclusions

Our results are a contribution to the comprehension of the risk level of the most northern population today known for this species, shedding light at a marginal range and ecological context. It appears that the studied peripheral population still maintains a high adaptive potential, due to both genetic and ecological factors. A relationship was found between fitness-related traits and environmental variables, such as mean temperatures of late winter-spring. The aggregated pattern in the distribution of individuals and their phenological traits is an expected consequence of facilitative processes in the recruitment process. Summarizing all results, the population displays a high phenotypic plasticity and is characterised by good levels of genetic variation. Therefore, *C. albidus* does not seem to be particularly disadvantaged by geographical and/or reproductive isolation, despite the great distance from the nearest currently known populations (ca. 250 km), which almost certainly impedes any genetic exchange between the Le Prandine population and the others. As a consequence, our case-study could be one of the numerous exceptions to the commonly accepted abundant centre hypothesis, also in light of what was reported by Abeli et al. (2014).

Currently, the study site is subject to the requirements of the conservation measures pursuant to the Habitat Directive (92/43/EEC) recently adopted by the Veneto Region (Regione del Veneto, 2017). Among these, the most relevant for the conservation of this species' habitat are those aimed at the maintenance of open lands and shrublands, and at limiting or controlling land conversion to urban and agricultural use. To be realistically taken and successful, those measures should be integrated into the existing regional planning system of forest and pastoral resources (Trentanovi et al., 2018).

Further research should deal with the analysis of land cover changes occurring in the area and their projection in the future. This will support the planning of sound biodiversity conservation strategies, including ecological and genetic data, and an assessment of future prospects of survival of other isolated or marginal populations of related species, also taking into account the costs of the required actions.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.flora.2019.02.003.

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